

98 Rec'd PCT/PTO 02 JAN 2002

FORM PTO-1390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

217677US0PCT

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/926853 JS

INTERNATIONAL APPLICATION NO.  
PCT/JP00/04348INTERNATIONAL FILING DATE  
30 June 2000PRIORITY DATE CLAIMED  
2 July 1999

TITLE OF INVENTION

DNA ENCODING SUCROSE PTS ENZYME II

APPLICANT(S) FOR DO/EO/US

IZUI Masako et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☒ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

PCT/IB/304/Drawings (2 sheets)

PCT/IB/308/Notice of Priority

Form PTO-1449/Form PTO-1595

Sequence Listing(14 pages)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) <b>10/019284</b>		INTERNATIONAL APPLICATION NO. <b>PCT/JP00/04348</b>		ATTORNEY'S DOCKET NUMBER <b>217677US0PCT</b>	
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24. The following fees are submitted: <b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :</b> <ul style="list-style-type: none"> <li><input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1040.00</b></li> <li><input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890.00</b></li> <li><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740.00</b></li> <li><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710.00</b></li> <li><input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b></li> </ul> <p style="text-align: center;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></p>				<b>\$890.00</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)). <input type="checkbox"/> 20. <input type="checkbox"/> 30				<b>\$0.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	3 - 20 =	0	x \$18.00	<b>\$0.00</b>	
Independent claims	2 - 3 =	0	x \$84.00	<b>\$0.00</b>	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$890.00</b>	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				<b>\$0.00</b>	
<b>SUBTOTAL =</b>				<b>\$890.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30 +				<b>\$0.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$890.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input checked="" type="checkbox"/>				<b>\$40.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$930.00</b>	
				<b>Amount to be:</b>	\$
				<b>charged</b>	\$


a.	<input checked="" type="checkbox"/>	A check in the amount of <u><b>\$930.00</b></u> to cover the above fees is enclosed.
b.	<input type="checkbox"/>	Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
c.	<input checked="" type="checkbox"/>	The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u><b>15-0030</b></u> . A duplicate copy of this sheet is enclosed.
d.	<input type="checkbox"/>	Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.

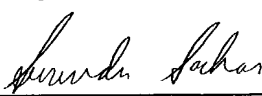
**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

**Surinder Sachar**  
Registration No. 34,423



**22850**

  
 SIGNATURE  
**Norman F. Oblon**  
 NAME  
**24,618**  
 REGISTRATION NUMBER  
Jan 2 2002  
 DATE

10019284 010202

10 RECEIVED 13 MAY 2002

217677USPCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :  
Masaka IZUI, et al. :  
SERIAL NO: 10/019,284 : ATTN: BOX SEQUENCE  
FILED: January 2, 2002 :  
FOR: DNA ENCODING SUCROSE PTS ENZYME II

PRELIMINARY AMENDMENT AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

In response to the Notice of March 11, 2002, Applicants respectfully submit herewith an amendment, Substitute Sequence Listing, and a computer-related Sequence Listing.

IN THE SPECIFICATION

At page 29, after the last line, on the next page, please delete the original Sequence Listing from pages 1-14 and insert the Substitute Sequence Listing attached hereto.

REMARKS

Claims 1-3 are pending.

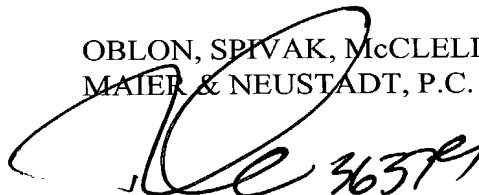
Contents of the paper copy of the Substitute Sequence Listing and the computer-readable Sequence Listing filed herewith are identical. Support for all the sequences listed in the Substitute Sequence Listing can be found in the present application. No new matter is

believed to be introduced by the submission of the Substitute Sequence Listing and the computer-readable Sequence Listing.


Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



Norman F. Oblon  
Attorney of Record  
Registration No. 24,618

 James J. Kelly, Ph.D.  
Registration No. 41,504



**22850**

(703) 413-3000  
Fax #: (703) 413-2220  
NFO/JK

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**Marked-Up Copy**

Serial No: 10/019,284

Amendment Filed on:

HEREWITH

IN THE SPECIFICATION

At page 29, after the last line, on the next page, please delete the original Sequence

Listing from pages 1-14 and insert the Substitute Sequence Listing attached hereto.

10 Recd

10019284 010202

09 DEC 2002

#13

217677US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :  
Masaka IZUI, et al. :  
SERIAL NO: 10/019,284 : ATTN: BOX SEQUENCE  
FILED: January 2, 2002 :  
FOR: DNA ENCODING SUCROSE PTS ENZYME II

PRELIMINARY AMENDMENT AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

In response to the Communication apparently dated September 24, 2002, Applicants respectfully submit herewith an amendment, Substitute Sequence Listing, and a substitute computer-readable Sequence Listing.

IN THE SPECIFICATION

At page 29, after the last line, on the next page, please delete the Sequence Listing filed May 13, 2002, and insert the Substitute Sequence Listing attached hereto.

REMARKS

Claims 1-3 are pending.

Applicants received the attached Communication from the USPTO with a fully torn cover sheet. Therefore, Applicants were unaware of the due date for replying to the Communication. Thereafter, Applicants attempted to contact Mark Spencer at the USPTO to

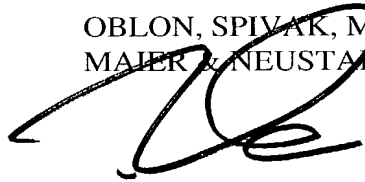
clarify the matter. Mark Spencer did not return our phone call until October 28, 2002. During this telephone conversation, Mark Spencer indicated that Applicants must contact OIPE at the USPTO to clarify any communication from the USPTO. Thereafter, Applicants contacted the general information desk at OIPE which indicated that the information about the Communication was unavailable. Applicants then contacted Ms. Erin Penders at the PCT department at the USPTO, who indicated that she has no record of the attached communication being mailed to Applicants. However, Ms. Penders indicated that she would retrieve the case and advise Applicants therefrom. On December 5, 2002, Applicants contacted Ms. Penders again regarding the matter. Although Ms. Penders indicated that she did not retrieve the case as of that date, she did indicate that the Communication may have been mailed September 24, 2002. To this date, Applicants have not been contacted by Ms. Penders while in possession of the case to establish the proper due date for the enclosed response. Therefore, Applicants enclose herewith, a substitute Sequence Listing and substitute computer readable Sequence Listing in response.

Contents of the paper copy of the Substitute Sequence Listing and the computer-readable Sequence Listing filed herewith are identical. Support for all the sequences listed in the Substitute Sequence Listing can be found in the present application. No new matter is believed to be introduced by the submission of the Substitute Sequence Listing and the computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.

 36378

Norman F. Oblon  
Attorney of Record  
Registration No. 24,618



James J. Kelly, Ph.D.  
Registration No. 41,504



**22850**

(703) 413-3000  
Fax #: (703) 413-2220  
NFO/JK

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217677US0PCT

**Marked-Up Copy**

Serial No: 10/019,284

Amendment Filed on:

HEREWITH

IN THE SPECIFICATION

--At page 29, after the last line, on the next page, please delete the Sequence Listing  
filed May 13, 2002, and insert the Substitute Sequence Listing attached hereto.--

2/PRTS

1

531 Rec'd PCT/IT

02 JAN 2002

## SPECIFICATION

DNA Encoding Sucrose PTS Enzyme II5 Technical Field

The present invention relates to a DNA encoding sucrose PTS enzyme II, which is a protein involved in uptake of sucrose into a cell of coryneform bacterium.

10 Background Art

Bacteria can assimilate many carbon sources, and various specific systems exist for their cellular transmembrane transport. Moreover, most of bacteria can respond to environmental changes to survive under a limited nutritious condition. Their cells are provided with a detector for monitoring the environment to select their nutrition from various carbon sources. Examples of such transmembrane transport systems and detectors of sugars include PTS (phosphoenolpyruvate/carbohydrate phosphotransferase system or phosphoenolpyruvate-sugar transport system; as for PTS, refer to *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Second Edition, ASM (American Society for Microbiology) Press).

PTS is involved in regulation of transmembrane transport and phosphorylation of various sugars (PTS sugars), movement towards these carbon sources and many metabolic pathways. PTS catalyzes the following

reaction. PEP refers to phosphoenolpyruvic acid.

PEP (intracellular) + Sugar (extracellular) -->

Pyruvic acid (intracellular) +

5                      Phosphorylated sugar (intracellular)

PTS catalyzes a reaction for generating a phosphorylated sugar and pyruvic acid by translocating a phosphate group of intracellular phosphoenolpyruvic acid  
10 (also referred to as "PEP" hereafter) to an extracellular sugar. The phosphorylation of a sugar is linked with cellular transmembrane transport of a sugar, and energies required for these processes are supplied from PEP, which is an intermediate of the glycolytic  
15 pathway.

In *Escherichia coli* and *Salmonella typhimurium*, proteins constituting PTS catalyze the following reactions.

- (1) PEP + EI --> P-EI + Pyruvic acid  
20 (2) P-EI + Hpr --> P-Hpr + EI  
(3) P-Hpr + EIIA --> P-EIIA + Hpr  
(4) P-EIIA + EIIB --> P-EIIB + EIIA  
(5) P-EIIB + Sugar (extracellular) + EIIB + Sugar-P  
(intracellular)

25                      Among proteins involved in the above reactions, EI (Enzyme I) and Hpr (histidine protein) are soluble cytoplasmic proteins involved in phosphorylation of all

PTS sugars and referred to as general PTS proteins.

On the other hand, EII (Enzyme II) is specific for PTS sugars and consists of several domains or proteins depending on the sugars. For example, the mannitol-specific EII is a membrane-bound protein consisting of three domains, A, B and C. The glucose-specific EII and sucrose-specific EII consist of IIB and IIC, which are membrane-bound proteins, and IIA, which is a soluble protein. In any case, translocation of a phosphate group from PEP to a sugar is mediated by EI, HPr, EIIA and EIIB. The EIIC domain, which is an intramembraneous portion of EII, forms a translocation channel and is considered to be probably a specific binding site of a substrate.

The third type of EII is observed in mannose PTS. Both of its domains A and B are fused in a single soluble polypeptide, and the two intramembraneous proteins (IIC and IID) are involved in transmembrane transport of mannose.

In *Escherichia coli* and *Salmonella typhimurium*, the gene encoding EI (ptsI) has been cloned and sequenced (Saffen, E.W. *et al.*, *J. Biol. Chem.*, 262, pp.16241-16253, 1987; De Reuse, H. and Danchin, A., *J. Bacteriol.*, 170, pp.3827-3837, 1988). Further, EII specific for some sugars have also been cloned (Saffen, E.W. *et al.*, *J. Biol. Chem.*, 262, pp.16241-16253, 1987; Erni, B. and Zanolari, B., *J. Biol. Chem.*, 261,

pp.16398-16403, 1986; Nelson, S.O. *et al.*, *EMBO J.*, 3, pp.1587-1593, 1984).

It is known that some kinds of sugars are taken up by non-PTS, which do not require PEP, as a system for uptake into cells.

#### Disclosure of the Invention

As described above, many studies about uptake of sugar into cells have been performed, but studies about PTS in industrially useful coryneform bacteria have not made much progress. Accordingly, an object of the present invention is to provide a gene encoding a protein constituting sucrose PTS in coryneform bacterium.

The inventors of the present application isolated a DNA fragment including a gene encoding sucrase (invertase) of coryneform bacterium and determined its structure. Further, they developed a method for producing an amino acid or a nucleic acid by using a coryneform bacterium containing the amplified sucrase gene (Japanese Patent Laid-open Publication (Kokai) Nos. 5-244958 and 8-196280). In the DNA fragment, four open reading frames (ORF-F1, ORF-F2, ORF-F3 and ORF-F4) exist in a *Sma*I fragment of about 6 kb.

However, the inventors of the present invention considered based on comparison with other sucrase genes that the aforementioned ORF-F2 did not contain the sucrase gene in full length. That is, the number of

amino acid residues in sucrase estimated from known  
sucrase genes is 466 to 511 (Gunaseakren, P., *J.*  
*Bacteriol.*, 172 (12), pp.6727-35, 1990), whereas the  
amino acid sequence that can be encoded by ORF-F2

5 contains 424 amino acid residues, which was relatively  
short. Therefore, a sequence existing downstream from  
ORF-F2 was cloned again and its nucleotide sequence was  
determined. As a result, it was revealed that the DNA  
fragment containing the aforementioned sucrase gene  
10 consisted of two independent cloned fragments ligated to  
each other, and it was found that a novel gene encoding  
sucrose PTS enzyme II existed downstream from the  
sucrase gene. Thus, the present invention was  
accomplished.

15 That is, the present invention provides a protein  
defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has the amino acid sequence of  
20 SEQ ID NO: 2 in Sequence Listing including substitution,  
deletion, insertion, addition or inversion of one or  
several amino acids, and an activity for binding to  
sucrose.

The present invention also provides a DNA which  
25 encodes a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and an activity for binding to sucrose.

The aforementioned DNA includes a DNA defined in the following (a) or (b):

(a) a DNA which contains the nucleotide sequence of the nucleotides 3779 to 5761 of SEQ ID NO: 1 in Sequence Listing;

(b) a DNA which is hybridizable with a nucleotide sequence containing the nucleotide sequence of the nucleotides 3779 to 5761 of SEQ ID NO: 1 in Sequence Listing under a stringent condition, and encodes a protein having an activity for binding to sucrose.

#### Brief Description of the Drawings

Fig. 1 shows a construction process of a plasmid for disrupting the sucrose PTS enzyme II gene.

Fig. 2 shows a construction process of pBCT4.

#### Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained in detail.

The DNA of the present invention was obtained, in the examples described later, by amplifying a region existing downstream from the sucrase gene on chromosomal

DNA of *Brevibacterium lactofermentum* by PCR (polymerase chain reaction).

A region adjacent to a known region on a chromosomal DNA can be amplified by ligating a cassette to a DNA fragment containing the regions and performing  
5 PCR using a primer corresponding to the known region and a primer corresponding to the cassette. At this time, if the 5' end of the cassette is dephosphorylated beforehand, a nick is generated at a ligation site of  
10 the chromosomal DNA fragment and 5' end of the cassette. Therefore, DNA synthesis started from the cassette primer will be stopped at this ligation site, and only the DNA synthesized from a synthetic primer will serve as a template for synthesis starting from the cassette  
15 primer and a complementary chain will be formed. As a result, specific amplification becomes possible (cassette-ligation mediated PCR method (*Molecular and Cellular Probes*, 6, pp.467-475)). A kit utilizing this method is commercially available (TAKARA LA PCR™ in  
20 vitro Cloning Kit, Takara Shuzo) and can be utilized to obtain the DNA of the present invention.

Since the nucleotide sequences of the DNA of the present invention and the adjacent region thereof have been revealed, they can be directly amplified by PCR  
25 using oligonucleotides synthesized based on these nucleotide sequences as primers and chromosomal DNA of coryneform bacterium as a template. Examples of such



primers include oligonucleotides having the nucleotide sequences of SEQ ID NOS: 10 and 21. Further, the DNA of the present invention can also be isolated from a chromosomal DNA library by hybridization using an  
5 oligonucleotide synthesized based on these nucleotide sequences as a probe. The chromosomal DNA of coryneform bacterium can be obtained by, for example, the method of Saito *et al.* (described in *Biochim. Biophys. Acta*, 72, pp.619-629, 1963) or the method of K. S. Kirby (*Biochem.*  
10 *J.*, 64, p.405, 1956).

Further, conventional methods well known to those skilled in the art can be employed for preparation of chromosomal DNA, preparation of chromosomal DNA library, hybridization, PCR, preparation of plasmid DNA,  
15 digestion and ligation of DNA, transformation, design of oligonucleotides used as primers and so forth. These methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press,  
20 1989 and so forth.

Plasmids used for cloning of the DNA of the present invention, preparation of a chromosomal DNA library or the like may be those that can replicate in microorganisms such as bacteria belonging to the genus  
25 *Escherichia*, and specific examples thereof include pBR322, pTWV228, pMW119, pUC19 and so forth.

An example of the nucleotide sequence of a DNA



depending on locations of amino acid residues in the three-dimensional structure of proteins and kinds of amino acid residues. This is due to the fact that there are highly analogous amino acids among amino acids such as isoleucine and valine, and difference among such amino acids does not substantially affect the three-dimensional structure of proteins. Therefore, the protein may be one having homology of 70% to 80% or higher, preferably, 90% to 95%, with respect to the whole amino acid sequence constituting sucrose PTS enzyme II and having an activity for binding to sucrose. Specifically, the term "several" means 2 to 180, preferably 2 to 60, more preferably 2 to 5.

Such a DNA encoding a protein which is substantially identical to sucrose PTS enzyme II as mentioned above can be obtained by modifying a nucleotide sequence so that the amino acid sequence at a particular site should include substitution, deletion, insertion, addition or inversion of an amino acid residue or residues through, for example, site-specific mutagenesis. Further, such a modified DNA as mentioned above may also be obtained by a conventional mutagenesis treatment. Examples of the mutagenesis treatment include an in vitro treatment of DNA encoding sucrose PTS enzyme II with hydroxylamine or the like, a treatment of microorganisms such as *Escherichia* bacteria containing the DNA encoding sucrose PTS enzyme II by UV

irradiation or with mutagenesis agents used for a usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and nitrous acid.

The aforementioned substitution, deletion,  
5 insertion, addition, inversion or the like of nucleotides includes naturally occurring mutations (mutant or variant) such as those observed depending on differences of strains, species or genera of microorganisms containing sucrose PTS enzyme II and so  
10 forth.

A DNA which encodes a protein substantially the same as sucrose PTS enzyme II can be obtained by, for example, isolating a DNA which is hybridizable with a DNA having the nucleotide sequence of the nucleotides  
15 3779 to 5761 of the nucleotide sequence of SEQ ID NO: 1 in Sequence Listing or a probe prepared from DNA having the nucleotide sequence by PCR or the like under stringent conditions and encoding a protein containing sucrose PTS enzyme II having an activity for binding to  
20 sucrose from DNA encoding sucrose PTS enzyme II containing a mutation or a cell containing it. The "stringent conditions" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to  
25 clearly define this condition by using numerical values. However, for example, the stringent conditions include a condition under which two of DNAs having high homology,

for example, two of DNAs having homology of not less than 50% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent conditions are exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization. The homology used herein is represented with a value calculated by the method of Lipman-Pearson (*Science*, 227, pp.1435-1441, 1985) or the method of Takashi & Gotoh (*J. Biochem.*, 92, pp.1173-1177, 1984). The probe can be designed according to a method known to those skilled in the art.

Those genes hybridizable under the condition as described above include those having a stop codon generated in the genes, but such genes can be easily removed by ligating them to a commercially available expression vector to examine size of the expressed product.

The protein of the present invention is a protein encoded by the DNA of the present invention and has the amino acid sequence of SEQ ID NO: 2. The protein of the present invention may have an amino acid sequence corresponding to the amino acid sequence of SEQ ID NO: 2 in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino

acids so long as it has an activity for binding to sucrose.

The DNA of the present invention can be utilized to improve sucrose uptake ability of coryneform bacteria or the like. Further, since PTS consumes PEP for uptake of a sugar into a cell, PTS is considered to be disadvantageous for synthesis of amino acids of which biosynthesis system include PEP in an upstream stage. Therefore, if sucrose PTS is disrupted and sucrose can be taken up by an uptake system which does not require PEP, it is considered advantageous in view of sucrose uptake rate or productivity of an amino acid or the like. In coryneform bacteria, non-PTS specific for sucrose is not known, but, for example, if sucrase is allowed to act extracellularly, glucose and fructose can be taken up by non-PTS.

Further, if the DNA of the present invention is modified so as to encode sucrose PTS enzyme II having an enhanced or suppressed function or so as to be ligated to an expression control sequence such as a promoter derived from other genes and introduced into a coryneform bacterium, a coryneform bacterium having an enhanced or suppressed sucrose uptake ability can be created. Specifically, a DNA encoding sucrose PTS enzyme II having an enhanced function is introduced into an autonomously replicable vector or chromosomal DNA in a cell of coryneform bacterium. Further, a DNA encoding

sucrose PTS enzyme II having a suppressed function is introduced into chromosomal DNA by gene substitution utilizing homologous recombination. Alternatively, a coryneform bacterium in which sucrose PTS functions at low temperature but does not function at high temperature can be created by gene substitution using a plasmid containing a temperature sensitive replication control region (see Japanese Patent Publication (Kokoku) No. 7-108228).

Coryneform bacteria to which the present invention is applicable include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, 41, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

*Corynebacterium acetoacidophilum*

*Corynebacterium acetoglutamicum*

*Corynebacterium alkanolyticum*

*Corynebacterium callunae*

*Corynebacterium glutamicum*

*Corynebacterium lilium* (*Corynebacterium glutamicum*)

*Corynebacterium melassecola*

*Corynebacterium thermoaminogenes*

*Corynebacterium herculis*

*Brevibacterium divaricatum* (*Corynebacterium glutamicum*)

*Brevibacterium flavum* (*Corynebacterium glutamicum*)

*Brevibacterium immariophilum*

5 *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*)

*Brevibacterium roseum*

*Brevibacterium saccharolyticum*

*Brevibacterium thiogenitalis*

10 *Brevibacterium ammoniagenes* (*Corynebacterium ammoniagenes*)

*Brevibacterium album*

*Brevibacterium cerium*

*Microbacterium ammoniaphilum*

15 Examples of the vector autonomously replicable in a cell of coryneform bacterium include pAM330 (refer to Japanese Patent Laid-open (Kokai) No. 58-67699), pHM1519 (refer to Japanese Patent Laid-open (Kokai) No. 58-77895) and so forth. Moreover, if a DNA fragment having  
20 an ability to make a plasmid autonomously replicable in coryneform bacterium is excised from these vectors and inserted into the vectors for *Escherichia coli*, they can be used as a so-called shuttle vector autonomously replicable in both of *Escherichia coli* and coryneform  
25 bacteria. Examples of such a shuttle vector include those mentioned below. There are also indicated microorganisms that harbor each vector, and accession



numbers thereof at international depositories are shown in the parentheses, respectively. Among these, pHSC4 includes a temperature sensitive replication control region.

5

pAJ655 *Escherichia coli* AJ11882 (FERM BP-136)  
*Corynebacterium glutamicum* SR8201 (ATCC39135)

pAJ1844 *Escherichia coli* AJ11883 (FERM BP-137)  
*Corynebacterium glutamicum* SR8202 (ATCC39136)

10

pAJ611 *Escherichia coli* AJ11884 (FERM BP-138)  
pAJ3148 *Corynebacterium glutamicum* SR8203 (ATCC39137)

pAJ440 *Bacillus subtilis* AJ11901 (FERM BP-140)

pHC4 *Escherichia coli* AJ12617 (FERM BP-3532)

pHSC4 *Escherichia coli* AJ12571 (FERM BP-3524)

15

A recombinant vector containing the DNA of the present invention can be introduced into a coryneform bacterium according to a transformation method reported so far. For instance, there are a method of treating  
20 recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *Escherichia coli* K-12 (Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159, 1970); and a method of preparing competent cells from cells which are at the growth phase  
25 followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene*, 1, 153, 1977). In

addition to these, also employable are a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing the recombinant DNA into the DNA-recipient cells, which method is known to be applicable to *Bacillus subtilis*, actinomycetes and yeasts (Chang, S. and Choen, S.N., *Molec. Gen. Genet.*, 168, 111, 1979; Bibb, M.J., Ward, J.M. and Hopwood, O.A., *Nature*, 274, 398, 1978; Hinnen, A., Hicks, J.B. and Fink, G.R., *Proc. Natl. Acad. Sci., USA*, 75, 1929, 1978) and the electric pulse method (see Japanese Patent Laid-open Publication (Kokai) No. 2-207791).

#### Examples

Hereafter, examples of the present invention will be explained in detail.

#### Example 1: Isolation of gene encoding sucrose PTS enzyme II

<1> Analysis of chromosomal DNA of *Brevibacterium lactofermentum* AJ12036 (FERM BP-734) by Southern hybridization

The *Brevibacterium lactofermentum* AJ12036 strain was cultured overnight in 4 ml of M-CM2S medium (containing 5 g/L of sucrose, 10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl and 0.1 g/L of DL-methionine) and microbial cells were collected.

Chromosomal DNA was extracted from the obtained microbial cells by using a Bacterial Geneomic DNA Purification Kit (Advanced Genetic Technologies). The chromosomal DNA was eluted with 50 µl of TE buffer  
5 (composition: 10 mM tris-HCl (pH 7.5), 1 mM EDTA-2Na).

The chromosomal DNA extracted as described above was subjected to Southern hybridization according to the method described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory  
10 Press, 1989. The chromosomal DNA was separately digested with *Bam*HI and *Sma*I, which did not cleave regions on the C-terminus side of ORF-F2 and N-terminus side of ORF-F3 and subjected to agarose gel electrophoresis. As a probe, a fragment of about 3 kb  
15 was used that was excised from 6.9 kb fragment cloned on pSSM30 (Japanese Patent Laid-open Publication (Kokai) No. 8-196280) with *Bam*HI to cover the regions on the C-terminus side of ORF-F2 and on the N-terminus side of ORF-F3 (Japanese Patent Laid-open Publication (Kokai) No.  
20 8-196280, the fragment of SEQ ID NO: 1649 to 4675 in Sequence Listing).

As a result of the hybridization, two bands were detected, and it was revealed that ORF-F2 and ORF-F3 were not adjacent to each other on the chromosome.  
25 Therefore, it was attempted to confirm a sequence existing downstream from the sucrase gene again.

<2> Determination of sequence of region existing downstream from sucrase gene

To determine the nucleotide sequence of a region downstream from the sucrase gene, the downstream region was first amplified by PCR. PCR was performed by using a TAKARA LA PCR™ in vitro Cloning Kit (Takara Shuzo). Specifically, the PCR was performed as follows.

The chromosomal DNA was completely digested with 10 kinds of restriction enzymes (*SpeI*, *EcoT14I*, *NheI*, *PstI*, *EcoT22I*, *BglIII*, *BamHI*, *XhoI*, *SallI*, *AvaI*), which produced the same cleavage ends as cassettes (SEQ ID NOS: 3 to 8 in Sequence Listing) attached to the aforementioned kit. PCR was performed by using each of these fragments as a template, Synthetic primer 1 shown in Table 1 and Cassette primer 1 (SEQ ID NO: 19). Since a phosphate group was not added to the 5' end of the cassette, a nick was generated at the ligation site of the chromosomal DNA fragment and the 5' end of the cassette. Therefore, the DNA synthesis starting from the cassette primer stopped at this ligation site, and only the DNA synthesized from the synthetic primer served as a template for synthesis starting from the cassette primer, and a complementary chain was formed.

Subsequently, PCR was performed out by using the amplification product obtained above as a template, Synthetic primer 2 and Cassette primer 2 (SEQ ID NO: 20). As a result, a fragment could be amplified when a DNA

obtained by digesting the chromosomal DNA with *Eco*T14I, *Pst*I, *Bgl*III, *Bam*HI, *Xho*I or *Ava*I was used as a template. The nucleotide sequence of a fragment of about 1.8 kb amplified by using the DNA fragment digested with *Bam*HI as a template was determined.

Table 1: Nucleotide sequence and position of synthetic primer

Primer number	Nucleotide sequence	Location in SEQ ID NO: 1 (nucleotide number)
1	CGTCTTGCGAGGATTCAGCGAGCTG (SEQ ID NO: 9)	(3159 to 3183)
2	AGCTGGATTTTCGGCCATGAATTCTA (SEQ ID NO: 10)	(3179 to 3203)
3	GATCTGTTCGGTCCGCAATCACT (SEQ ID NO: 11)	(4189 to 4212)
4	CACTGGTGGAGATGTTCCCTCAGAT (SEQ ID NO: 12)	(4209 to 4233)
5	CATCTTCGCAACCGCATCCATGGCC (SEQ ID NO: 13)	(4801 to 4825)
6	CGCGCAGGGTGCAGCATGTTTGCC (SEQ ID NO: 14)	(4831 to 4854)
7	GGGCCTTGCAGGTGCTTCAGGTGTC (SEQ ID NO: 15)	(4888 to 4912)
8	CCGCTGTTCTTGGTATTACAGAGCC (SEQ ID NO: 16)	(4914 to 4938)
9	GCAGCGTCAGCGATGCCATGTTTGC (SEQ ID NO: 17)	(5322 to 5346)
10	GCTTGGCTCAGGTGTTGCGATCGTC (SEQ ID NO: 18)	(5356 to 5380)

Synthetic primers 3 and 4 were synthesized based on the determined sequences. In the same manner as described above, the fragments were successively amplified by PCR using a combination of Synthetic primer 3 and Cassette primer 1 and a combination of Synthetic

primer 4 and Cassette primer 2. As a result, a fragment could be amplified when a DNA obtained by digesting the chromosomal DNA with *Pst*I or *Bam*HI was used as a template. The nucleotide sequence of the fragment amplified based on the DNA fragment digested with *Pst*I was determined.

Synthetic primers 5 and 6 were synthesized based on the determined sequence. PCR was successively carried out by using a combination of Synthetic primer 5 and Cassette primer 1 and a combination of Synthetic primer 6 and Cassette primer 2. As a result, an amplified fragment could be confirmed when the chromosomal DNA digested with *Eco*T14 or *Pst*I was used as a template. The nucleotide sequence of the fragment of the former case was determined.

Further, Synthetic primers 7 and 8 were synthesized and the same procedure as described above was performed. As a result, an amplified fragment could be confirmed when *Eco*T14-digested chromosomal DNA was used as a template. The nucleotide sequence of this amplified fragment was determined.

Primers 9 and 10 were synthesized based on the above sequence, and the same procedure as described above was performed. As a result, an amplified fragment could be confirmed when *Spe*I-digested chromosomal DNA was used as a template. The nucleotide sequence of this amplified fragment was determined.

As for the nucleotide sequence determination, a reaction was performed by using a sequencing kit produced by ABI according to its protocol, and then the nucleotide sequence of the amplified fragment was  
5 determined by the fluorescence labeling method.

The above results are shown in SEQ ID NO: 1 in Sequence Listing. It was found that a novel ORF existed after the nucleotide number 3684 in this nucleotide sequence. It was inferred that this ORF consisted of  
10 the nucleotide sequence of 1983 bp corresponding to the nucleotide numbers 3779 to 5761, and that a protein obtained by translating the determined nucleotide sequence consisted of 661 amino acids. As for the ORF, homology search was performed in the GENBANK CDS  
15 database. As a result, as shown in Table 2, the proteins that could be encoded by the ORF showed high homology with sucrose PTS enzyme II, a protein specific for sucrose uptake. Hereafter, this ORF is referred to as ptsIIsuc gene.

20

25

Table 2: Results of homology search of novel ORF

Name of Bacterium and gene		Known protein showing homology	Homology (%)
<i>P. pentsaceus</i>	scrA	Enzyme IIscr	48.8
<i>B. subtilis</i>	treP	Trehalose-specific enzyme IIBC	43.4
<i>S. xylosus</i>	scrA	Enzyme IIscr	52.2
<i>S. mutans</i>	scrA	Enzyme IIscr	45.4
<i>S. typhimurium</i>	scrA	Enzyme Iiscr	37.6
plasmid pUR400			

Example 2: Preparation of sucrose PTS enzyme II gene-disrupted strain

5           A *Brevibacterium lactofermentum* strain with a disrupted ptsIIIsuc gene was prepared. First of all, a plasmid for disrupting the gene was prepared (Fig. 1). A ptsIIIsuc gene fragment amplified by PCR using the chromosome of *Brevibacterium lactofermentum* AJ12036 as a

10       template, Primer 2 (SEQ ID NO: 10) and Primer 11 (SEQ ID NO: 21) having the nucleotide sequence shown below was cloned by using a TA cloning kit (Invitrogen), and the plasmid was designated as pCRS2.

15       (Primer 11)  
CGCTACTGCTGAACGAACATGTCC (corresponding to the nucleotide numbers 5947 to 5924 in SEQ ID NO: 1)

20           A fragment excised from pCRS2 by digestion with *Xba*I and *Spe*I was ligated to the *Xba*I ends of pHSG399 to construct p399S2. This plasmid was digested with *Hpa*I and *Bam*HI, and the obtained fragment (corresponding to



the nucleotide numbers 4385 to 4798 in SEQ ID NO: 1) was  
ligated to pHSG299 digested with *Sma*I and *Bam*HI to  
prepare a plasmid pdSB. Subsequently, pdSB was digested  
with *Bam*HI and ligated to a temperature sensitive  
5 replication origin that was excised from plasmid pBCT4  
by digestion with *Bam*HI and could replicate in  
coryneform bacteria (refer to Japanese Patent  
Publication (Kokoku) No. 7-108228) to prepare a plasmid  
pdSBT. The plasmid included the ptsII<sub>suc</sub> gene having  
10 deleted 5' end and 3' ends. The pdSBT could autonomously  
replicate in coryneform bacteria at about 10°C to 32°C,  
but not at about 34°C or higher.

The pBCT4 was constructed as follows. A  
temperature sensitive vector pHSC4 described in Japanese  
15 Patent Publication (Kokoku) No. 7-108228 was digested  
with restriction enzymes, *Bam*HI and *Kpn*I, to obtain a  
DNA fragment of about 3 kb containing the obtained  
temperature sensitive replication origin. Both of the  
ends of the obtained DNA fragment were blunt-ended with  
20 T4 DNA polymerase. This DNA fragment was ligated with  
*Bam*HI linkers and digested with *Bam*HI again. Then, it  
was ligated to pHSG399 digested with *Bam*HI to obtain  
pBCT4 (Fig. 2).

The *Brevibacterium lactofermentum* AJ12036 strain  
25 was transformed with pdSBT and a transformant was  
selected by using a CM2S plate containing 25 µg/ml of  
kanamycin. The transformation was performed by the

electric pulse method (refer to Japanese Patent Laid-open Publication (Kokai) No. 2-207791). The obtained transformant was designated as AJ12036/pTSBT. The AJ12036/pTSBT strain was diluted and spread on M-CM2S  
5 plates containing 25 µg/ml of kanamycin at  $10^3$  to  $10^5$  cfu per plate. The transformants on the plates were cultured overnight at 34°C, and a strain showing drug resistance was obtained as a strain containing the plasmid incorporated into its chromosome. It was  
10 confirmed by PCR that the obtained strain had the vector plasmid incorporated into the ptsIIIsuc gene of the host chromosome by homologous recombination. This integrated strain was designated as YdS1.

The YdS1 strain was cultured at 34°C in a minimal  
15 medium containing glucose or sucrose as a sugar source (20g/L of glucose or sucrose, 5 g/L of ammonium sulfate, 2 g/L of urea, 1 g/L of  $\text{KH}_2\text{PO}_4$ , 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.002 g/dl of  $\text{FeSO}_4$ , 0.002 g/dl of  $\text{MnSO}_4$ , 100 µg/L of biotin, 2000 µg/L of vitamin B1, 10 mg/dl of DL-  
20 methionine and 15 g/L of agar, pH 6.6). The results are shown in Table 3. Since the YdS1 strain could grow in the minimal medium containing only glucose as a carbon source, but not in the minimal medium containing only sucrose as a carbon source, it was confirmed that the  
25 ptsIIIsuc gene is the gene encoding Enzyme II which is a protein specific for sucrose in sucrose uptake.

Table 3: Growth on minimal medium

Bacterial strain	Carbon source	
	Sucrose	Glucose
AJ12036	Possible to grow	Possible to grow
YdS1	Impossible to grow	Possible to grow

#### Industrial Applicability

5           The present invention provides a gene encoding  
sucrose PTS enzyme II of coryneform bacterium and a  
strain of coryneform bacterium in which sucrose PTS does  
not function. These gene and bacterial strain can be  
utilized in breeding of strains with improved sugar  
10 uptake rate or improved productivity of an amino acid, a  
nucleic acid or the like.

What is claimed is:

1. A protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing;

5 (B) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing including substitution,  
deletion, insertion, addition or inversion of one or  
several amino acids, and an activity for binding to  
sucrose.

10

2. A DNA which encodes a protein defined in the  
following (A) or (B):

(A) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing;

15 (B) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing including substitution,  
deletion, insertion, addition or inversion of one or  
several amino acids, and an activity for binding to  
sucrose.

20

3. The DNA according to Claim 2, which is a DNA  
defined in the following (a) or (b):

(a) a DNA which contains the nucleotide sequence  
of the nucleotides 3779 to 5761 of SEQ ID NO: 1 in  
25 Sequence Listing;

(b) a DNA which is hybridizable with a nucleotide  
sequence containing the nucleotide sequence of the

nucleotides 3779 to 5761 of SEQ ID NO: 1 in Sequence  
 Listing under stringent conditions, and encodes a  
 protein having an activity for binding to sucrose.

## ABSTRACT

A gene encoding a protein constituting sucrose PTS  
of coryneform bacterium is provided by amplifying a  
5 region existing downstream from sucrase gene in  
coryneform bacterium by the cassette-ligation mediated  
PCR to obtain DNA encoding sucrose PTS enzyme II, which  
is a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of  
10 SEQ ID NO: 2 in Sequence Listing;

(B) a protein which has the amino acid sequence of  
SEQ ID NO: 2 in Sequence Listing including substitution,  
deletion, insertion, addition or inversion of one or  
several amino acids, and an activity for binding to  
15 sucrose.

1/2

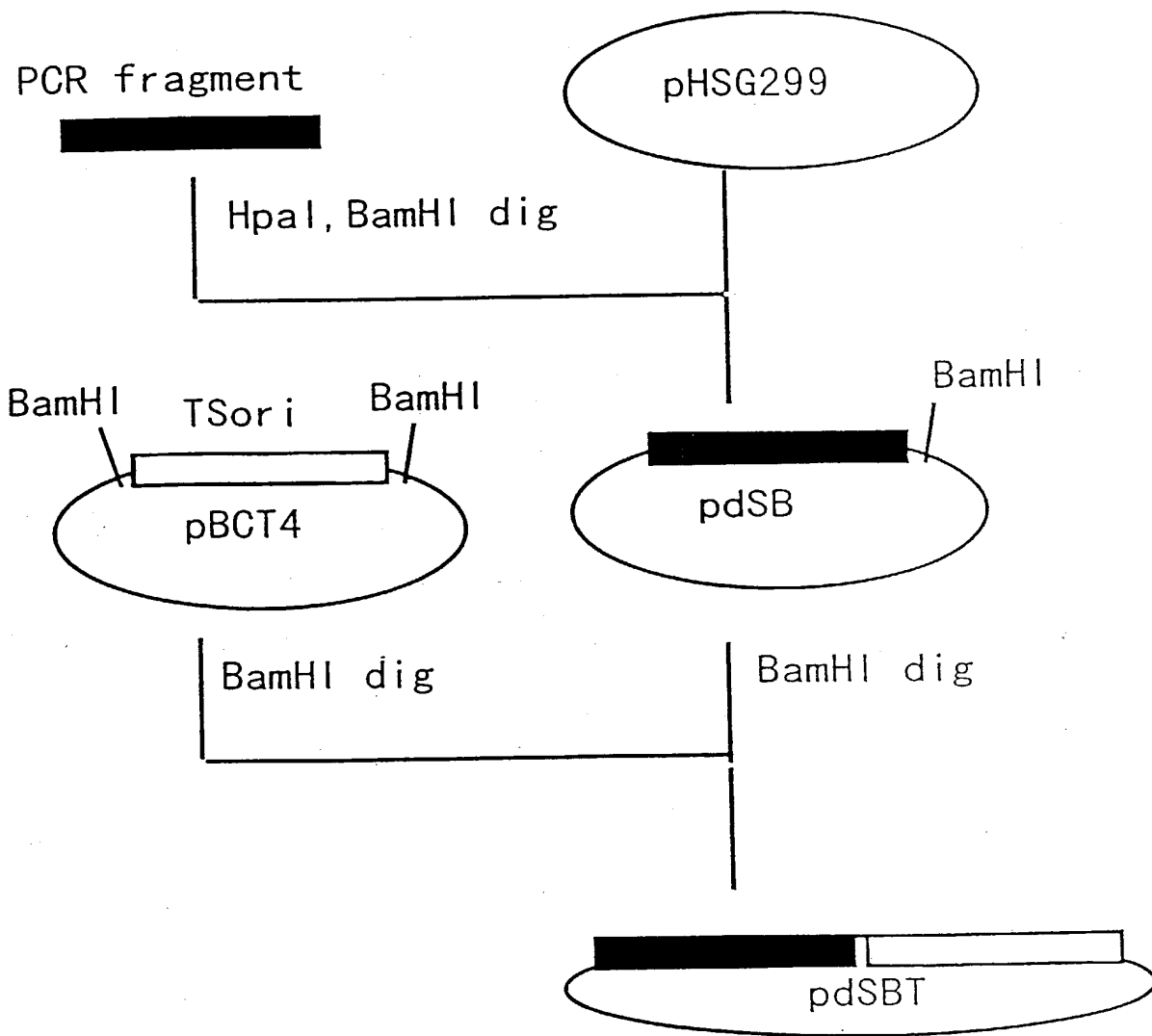


FIG.1

2/2

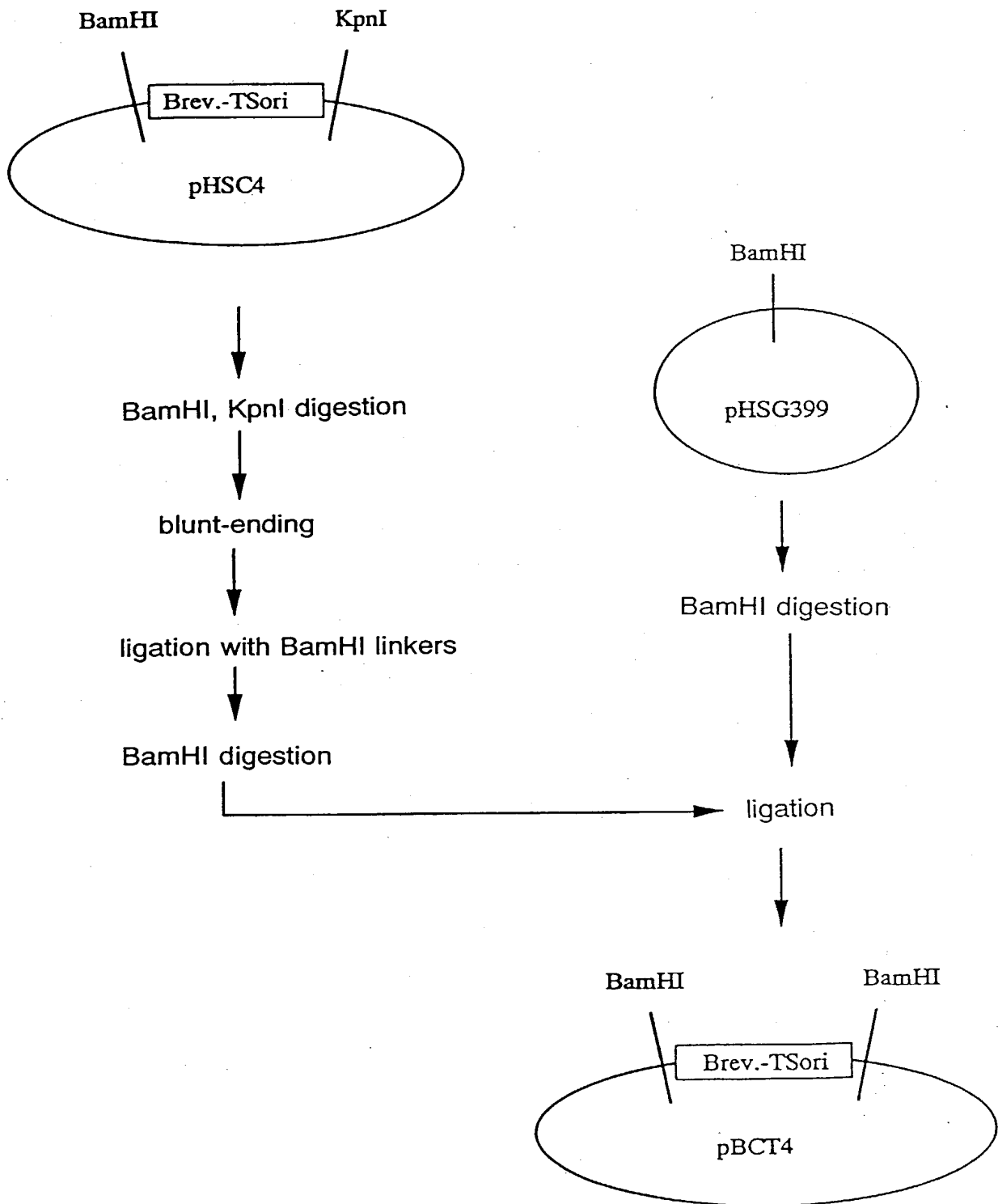


FIG.2



# Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

DNA Encoding Sucrose PTS Enzyme II

the specification of which

☐ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and amended on \_\_\_\_\_.

☒ was filed as PCT international application

Number PCT/JP 00/04348

on June 30, 2000,

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
11-189512	Japan	02/07/1999	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.

Filing Date

Status (pending, patented,  
abandoned)

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Masako IZUI

NAME OF FIRST SOLE INVENTOR

Residence: Kawasaki-shi, Kanagawa, Japan JPXMasako Izui

Signature of Inventor

Citizen of: Japan

Post Office Address:

c/o Ajinomoto Co., Inc., Fermentation &  
Biotechnology Laboratories, 1-1, Suzuki-cho,  
Kawasaki-ku, Kawasaki-shi, Kanagawa,  
210-8681 Japan

DEcember 10, 2001

Date

2 Masakazu SUGIMOTO

NAME OF SECOND JOINT INVENTOR

Masakazu Sugimoto  
Signature of Inventor

December 10, 2001

3 Date

Tsuyoshi NAKAMATSU

NAME OF THIRD JOINT INVENTOR

Tsuyoshi Nakamatsu  
Signature of Inventor

December 10, 2001

4 Date

Osamu KURAHASHI

NAME OF FOURTH JOINT INVENTOR

Osamu Kurahashi  
Signature of Inventor

December 10, 2001

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence: Kawasaki-shi, Kanagawa, Japan JPX

Citizen of: Japan

Post Office Address:

c/o Ajinomoto Co., Inc., Fermentation &  
Biotechnology Laboratories, 1-1, Suzuki-cho,  
Kawasaki-ku, Kawasaki-shi, Kanagawa,  
210-8681 Japan

Residence: Kawasaki-shi, Kanagawa, Japan JPX

Citizen of: Japan

Post Office Address:

c/o Ajinomoto Co., Inc., Fermentation &  
Biotechnology Laboratories, 1-1, Suzuki-cho,  
Kawasaki-ku, Kawasaki-shi, Kanagawa,  
210-8681 Japan

Residence: Kawasaki-shi, Kanagawa, Japan JPX

Citizen of: Japan

Post Office Address:

c/o Ajinomoto Co., Inc., Fermentation &  
Biotechnology Laboratories, 1-1, Suzuki-cho,  
Kawasaki-ku, Kawasaki-shi, Kanagawa,  
210-8681 Japan

10019284.010202

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<sup>1</sup>531 Rec'd PCT. 02 JAN 2002

SEQUENCE LISTING

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<150> JP 11-189512

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 ttttgaacaa acattcatgt ctgaatatit ttgtttcttc ccggttaagg agaaattc 3778  
 atg gac cat aag gac ctc gcg caa cgc atc ctg cgc gac att ggc ggc 3826  
 Met Asp His Lys Asp Leu Ala Gln Arg Ile Leu Arg Asp Ile Gly Gly  
 1 5 10 15  
 gaa gac aac att gtc gcc gcc gca cac tgt gca acg cgt tta cgc ctc 3874  
 Glu Asp Asn Ile Val Ala Ala Ala His Cys Ala Thr Arg Leu Arg Leu  
 20 25 30  
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 Val Leu Lys Asp Thr Lys Asp Val Asp Arg Gln Ser Leu Asp Asp Asp  
 35 40 45  
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 Pro Asp Leu Lys Gly Thr Phe Glu Thr Gly Gly Met Phe Gln Ile Ile  
 50 55 60  
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 Val Gly Pro Gly Asp Val Asp His Val Phe Lys Glu Leu Asp Asp Ala  
 65 70 75 80  
 acc tcc aaa gac atc gct gtg tcc aca gag cag ctc aaa gat gtt gtg 4066  
 Thr Ser Lys Asp Ile Ala Val Ser Thr Glu Gln Leu Lys Asp Val Val  
 85 90 95  
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 Ala Asn Asn Ala Asn Trp Phe Ser Arg Ala Val Lys Val Leu Ala Asp  
 100 105 110  
 att ttc gtc ccg ctg att cca atc ttg gtt ggt ggc ggt ctg ctc atg 4162  
 Ile Phe Val Pro Leu Ile Pro Ile Leu Val Gly Gly Gly Leu Leu Met  
 115 120 125  
 gct atc aac aat gtg ttg gtt gcg cag gat ctg ttc ggt ccg caa tca 4210  
 Ala Ile Asn Asn Val Leu Val Ala Gln Asp Leu Phe Gly Pro Gln Ser  
 130 135 140  
 ctg gtg gag atg ttc cct cag atc agc ggt gtt gct gag atg atc aac 4258  
 Leu Val Glu Met Phe Pro Gln Ile Ser Gly Val Ala Glu Met Ile Asn  
 145 150 155 160  
 ctg atg gca tct gcg ccg ttc gcg ttc ttg cca gtg ttg gtt ggt ttc 4306  
 Leu Met Ala Ser Ala Pro Phe Ala Phe Leu Pro Val Leu Val Gly Phe  
 165 170 175

acc gca acc aag cgt ttc ggt ggc aat gag ttc ctg ggc gcc ggc att 4354  
 Thr Ala Thr Lys Arg Phe Gly Gly Asn Glu Phe Leu Gly Ala Gly Ile  
                   180                  185                  190  
 ggt atg gcg atg gtg ttc cca acc ctg gtt aac ggc tac gac gtg gcc 4402  
 Gly Met Ala Met Val Phe Pro Thr Leu Val Asn Gly Tyr Asp Val Ala  
                   195                  200                  205  
 gcc acc atg acc gcg ggc gaa atg cca atg tgg tcc ctg ttt ggt ttg 4450  
 Ala Thr Met Thr Ala Gly Glu Met Pro Met Trp Ser Leu Phe Gly Leu  
                   210                  215                  220  
 gat gtt gct caa gct ggt tac cag ggc acc gtg ctt cct gtg ctg gtg 4498  
 Asp Val Ala Gln Ala Gly Tyr Gln Gly Thr Val Leu Pro Val Leu Val  
 225                  230                  235                  240  
 gtc tct tgg att ctg gca acg atc gag aag ttc ctg cac aag cga ctc 4546  
 Val Ser Trp Ile Leu Ala Thr Ile Glu Lys Phe Leu His Lys Arg Leu  
                   245                  250                  255  
 atg ggc act gca gac ttc ctg atc acc cca gtg ttg act ctg ctg ctc 4594  
 Met Gly Thr Ala Asp Phe Leu Ile Thr Pro Val Leu Thr Leu Leu Leu  
                   260                  265                  270  
 acc ggc ttc ctt acg ttc att gct att ggt cca gca atg cgc tgg gtg 4642  
 Thr Gly Phe Leu Thr Phe Ile Ala Ile Gly Pro Ala Met Arg Trp Val  
                   275                  280                  285  
 ggt gac ttg ctg gca cac ggt ctg cag gga ctc tat gat ttc ggt ggt 4690  
 Gly Asp Leu Leu Ala His Gly Leu Gln Gly Leu Tyr Asp Phe Gly Gly  
                   290                  295                  300  
 cca gtc ggc ggt ctg ctt ttc ggt ctg gtc tac tca cca atc gtt atc 4738  
 Pro Val Gly Gly Leu Leu Phe Gly Leu Val Tyr Ser Pro Ile Val Ile  
 305                  310                  315                  320  
 act ggt ctg cac cag tcc ttc ccg cca att gag ctg gag ctg ttc aac 4786  
 Thr Gly Leu His Gln Ser Phe Pro Pro Ile Glu Leu Glu Leu Phe Asn  
                   325                  330                  335  
 cag ggt gga tcc ttc atc ttc gca acc gca tcc atg gcc aat atc gcg 4834  
 Gln Gly Gly Ser Phe Ile Phe Ala Thr Ala Ser Met Ala Asn Ile Ala  
                   340                  345                  350  
 cag ggt gca gca tgt ttg gca gtg ttc ttc cta gcg aag agt gaa aag 4882  
 Gln Gly Ala Ala Cys Leu Ala Val Phe Phe Leu Ala Lys Ser Glu Lys  
                   355                  360                  365  
 ctc aag ggc ctt gca ggt gct tca ggt gtc tcc gct gtt ctt ggt att 4930  
 Leu Lys Gly Leu Ala Gly Ala Ser Gly Val Ser Ala Val Leu Gly Ile  
                   370                  375                  380  
 aca gag cct gcg atc ttc ggt gtg aac ctt cgc ctg cgc tgg ccg ttc 4978  
 Thr Glu Pro Ala Ile Phe Gly Val Asn Leu Arg Leu Arg Trp Pro Phe

385	390	395	400	
tac att ggt atc ggt acc gca gct atc ggt ggc gct ttg att gca ctc				5026
Tyr Ile Gly Ile Gly Thr Ala Ala Ile Gly Gly Ala Leu Ile Ala Leu				
	405	410	415	
ttt gat atc aag gca gtt gcg ttg ggc gct gca ggt ttc ttg ggt gtt				5074
Phe Asp Ile Lys Ala Val Ala Leu Gly Ala Ala Gly Phe Leu Gly Val				
	420	425	430	
gtt tct att gat gct cca gat atg gtc atg ttc ttg gtt tgc gcg gta				5122
Val Ser Ile Asp Ala Pro Asp Met Val Met Phe Leu Val Cys Ala Val				
	435	440	445	
gtt acc ttt gtc atc gca ttc ggc gca gcg att gct tat ggc ctt tac				5170
Val Thr Phe Val Ile Ala Phe Gly Ala Ala Ile Ala Tyr Gly Leu Tyr				
	450	455	460	
ttg gtt cgc cgc aac ggc agc att gat cca gat gca acc gct gct cca				5218
Leu Val Arg Arg Asn Gly Ser Ile Asp Pro Asp Ala Thr Ala Ala Pro				
465	470	475	480	
gtg cct gca gga acg acc aaa gcc gaa gca gaa gca ccc gca gaa ttt				5266
Val Pro Ala Gly Thr Thr Lys Ala Glu Ala Glu Ala Pro Ala Glu Phe				
	485	490	495	
tca aac gat tcc acc atc atc cag gca cct ttg acc ggt gaa gct atc				5314
Ser Asn Asp Ser Thr Ile Ile Gln Ala Pro Leu Thr Gly Glu Ala Ile				
	500	505	510	
gca ctg agc agc gtc agc gat gcc atg ttt gcc agc gga aag ctt ggc				5362
Ala Leu Ser Ser Val Ser Asp Ala Met Phe Ala Ser Gly Lys Leu Gly				
	515	520	525	
tca ggt gtt gcg atc gtc ccc acc aag ggg cag ctg gtt tca cca gtg				5410
Ser Gly Val Ala Ile Val Pro Thr Lys Gly Gln Leu Val Ser Pro Val				
	530	535	540	
agc gga aag atc gtg gtg gcc ttc cca tct ggt cac gct ttc gca gtc				5458
Ser Gly Lys Ile Val Val Ala Phe Pro Ser Gly His Ala Phe Ala Val				
545	550	555	560	
cgc act aag gct gag gat ggt tcc aat gtg gat atc ttg atg cac att				5506
Arg Thr Lys Ala Glu Asp Gly Ser Asn Val Asp Ile Leu Met His Ile				
	565	570	575	
ggt ttc gac acc gta aac ctc aac ggc acg cac ttt aac ccg ctg aag				5554
Gly Phe Asp Thr Val Asn Leu Asn Gly Thr His Phe Asn Pro Leu Lys				
	580	585	590	
aag cag ggc gat gaa gtc aaa gca ggg gag ctg ctg tgt gaa ttc gat				5602
Lys Gln Gly Asp Glu Val Lys Ala Gly Glu Leu Leu Cys Glu Phe Asp				
	595	600	605	
att gat gcc att aag gct gca ggt tat gag gta acc acg ccg att gtt				5650



Ile Asp Ala Ile Lys Ala Ala Gly Tyr Glu Val Thr Thr Pro Ile Val  
 610 615 620  
 gtt tcg aat tac aag aaa acc gga cct gta aac act tac ggt ttg ggc 5698  
 Val Ser Asn Tyr Lys Lys Thr Gly Pro Val Asn Thr Tyr Gly Leu Gly  
 625 630 635 640  
 gaa att gaa gcg gga gcc aac ctg ctc aac gtc gca aag aaa gaa gcg 5746  
 Glu Ile Glu Ala Gly Ala Asn Leu Leu Asn Val Ala Lys Lys Glu Ala  
 645 650 655  
 gtg cca gca aca cca taagttgaaa ccttgagtgt tcgcacacag gttagactag 5801  
 Val Pro Ala Thr Pro  
 660  
 gggacgtgac tctacgcac tttgacaccg gtaccggtac gcttcgagat tttaaacctg 5861  
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&lt;210&gt; 2

&lt;211&gt; 661

&lt;212&gt; PRT

&lt;213&gt; Brevibacterium lactofermentum

&lt;400&gt; 2

Met Asp His Lys Asp Leu Ala Gln Arg Ile Leu Arg Asp Ile Gly Gly  
 1 5 10 15  
 Glu Asp Asn Ile Val Ala Ala Ala His Cys Ala Thr Arg Leu Arg Leu  
 20 25 30  
 Val Leu Lys Asp Thr Lys Asp Val Asp Arg Gln Ser Leu Asp Asp Asp  
 35 40 45  
 Pro Asp Leu Lys Gly Thr Phe Glu Thr Gly Gly Met Phe Gln Ile Ile  
 50 55 60  
 Val Gly Pro Gly Asp Val Asp His Val Phe Lys Glu Leu Asp Asp Ala  
 65 70 75 80  
 Thr Ser Lys Asp Ile Ala Val Ser Thr Glu Gln Leu Lys Asp Val Val  
 85 90 95  
 Ala Asn Asn Ala Asn Trp Phe Ser Arg Ala Val Lys Val Leu Ala Asp  
 100 105 110  
 Ile Phe Val Pro Leu Ile Pro Ile Leu Val Gly Gly Gly Leu Leu Met  
 115 120 125  
 Ala Ile Asn Asn Val Leu Val Ala Gln Asp Leu Phe Gly Pro Gln Ser  
 130 135 140  
 Leu Val Glu Met Phe Pro Gln Ile Ser Gly Val Ala Glu Met Ile Asn  
 145 150 155 160

Leu Met Ala Ser Ala Pro Phe Ala Phe Leu Pro Val Leu Val Gly Phe  
 165 170 175  
 Thr Ala Thr Lys Arg Phe Gly Gly Asn Glu Phe Leu Gly Ala Gly Ile  
 180 185 190  
 Gly Met Ala Met Val Phe Pro Thr Leu Val Asn Gly Tyr Asp Val Ala  
 195 200 205  
 Ala Thr Met Thr Ala Gly Glu Met Pro Met Trp Ser Leu Phe Gly Leu  
 210 215 220  
 Asp Val Ala Gln Ala Gly Tyr Gln Gly Thr Val Leu Pro Val Leu Val  
 225 230 235 240  
 Val Ser Trp Ile Leu Ala Thr Ile Glu Lys Phe Leu His Lys Arg Leu  
 245 250 255  
 Met Gly Thr Ala Asp Phe Leu Ile Thr Pro Val Leu Thr Leu Leu Leu  
 260 265 270  
 Thr Gly Phe Leu Thr Phe Ile Ala Ile Gly Pro Ala Met Arg Trp Val  
 275 280 285  
 Gly Asp Leu Leu Ala His Gly Leu Gln Gly Leu Tyr Asp Phe Gly Gly  
 290 295 300  
 Pro Val Gly Gly Leu Leu Phe Gly Leu Val Tyr Ser Pro Ile Val Ile  
 305 310 315 320  
 Thr Gly Leu His Gln Ser Phe Pro Pro Ile Glu Leu Glu Leu Phe Asn  
 325 330 335  
 Gln Gly Gly Ser Phe Ile Phe Ala Thr Ala Ser Met Ala Asn Ile Ala  
 340 345 350  
 Gln Gly Ala Ala Cys Leu Ala Val Phe Phe Leu Ala Lys Ser Glu Lys  
 355 360 365  
 Leu Lys Gly Leu Ala Gly Ala Ser Gly Val Ser Ala Val Leu Gly Ile  
 370 375 380  
 Thr Glu Pro Ala Ile Phe Gly Val Asn Leu Arg Leu Arg Trp Pro Phe  
 385 390 395 400  
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 Phe Asp Ile Lys Ala Val Ala Leu Gly Ala Ala Gly Phe Leu Gly Val  
 420 425 430  
 Val Ser Ile Asp Ala Pro Asp Met Val Met Phe Leu Val Cys Ala Val  
 435 440 445  
 Val Thr Phe Val Ile Ala Phe Gly Ala Ala Ile Ala Tyr Gly Leu Tyr  
 450 455 460  
 Leu Val Arg Arg Asn Gly Ser Ile Asp Pro Asp Ala Thr Ala Ala Pro  
 465 470 475 480  
 Val Pro Ala Gly Thr Thr Lys Ala Glu Ala Glu Ala Pro Ala Glu Phe

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Ser Asn Asp	Ser Thr Ile Ile Gln Ala Pro Leu Thr Gly Glu Ala Ile				
	500		505		510
Ala Leu Ser Ser Val Ser Asp Ala Met Phe Ala Ser Gly Lys Leu Gly					
	515		520		525
Ser Gly Val Ala Ile Val Pro Thr Lys Gly Gln Leu Val Ser Pro Val					
	530		535		540
Ser Gly Lys Ile Val Val Ala Phe Pro Ser Gly His Ala Phe Ala Val					
545		550		555	560
Arg Thr Lys Ala Glu Asp Gly Ser Asn Val Asp Ile Leu Met His Ile					
	565		570		575
Gly Phe Asp Thr Val Asn Leu Asn Gly Thr His Phe Asn Pro Leu Lys					
	580		585		590
Lys Gln Gly Asp Glu Val Lys Ala Gly Glu Leu Leu Cys Glu Phe Asp					
	595		600		605
Ile Asp Ala Ile Lys Ala Ala Gly Tyr Glu Val Thr Thr Pro Ile Val					
	610		615		620
Val Ser Asn Tyr Lys Lys Thr Gly Pro Val Asn Thr Tyr Gly Leu Gly					
625		630		635	640
Glu Ile Glu Ala Gly Ala Asn Leu Leu Asn Val Ala Lys Lys Glu Ala					
	645		650		655
Val Pro Ala Thr Pro					
	660				

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<211> 44

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Sau3AI cassette

<220>

<221> misc\_feature

<222> (44)

<223> complementary strand extends a single strand having  
a sequence of 3'-ctag-5' at this position in the  
direction of 5' from 3'

<400> 3

gtacatattg tcgttagaac gcgtaatacg actcactata ggga

<210> 4  
<211> 47  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: EcoRI cassette

<220>  
<221> misc\_feature  
<222> (47)  
<223> complementary strand extends a single strand having  
a sequence of 3'-ttaa-5' at this position in the  
direction of 5' from 3'

<400> 4  
gtacatattg tcgtagaac gcgtaatacg actcactata gggagag 47

<210> 5  
<211> 46  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: HindIII cassette

<220>  
<221> misc\_feature  
<222> (46)  
<223> complementary strand extends a single strand having  
a sequence of 3'-tcga-5' at this position in the  
direction of 5' from 3'

<400> 5  
gtacatattg tcgtagaac gcgtaatacg actcactata gggaga 46

<210> 6  
<211> 51  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PstI cassette

<220>

<221> misc\_feature

<222> (48)..(51)

<223> complementary strand does not exist

<400> 6

gtacatattg tcgttagaac gcgtaatacg actcactata gggagactgc a

51

<210> 7

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: SalI cassette

<220>

<221> misc\_feature

<222> (47)

<223> complementary strand extends a single strand having  
a sequence of 3'-agct-5' at this position in the  
direction of 5' from 3'

<400> 7

gtacatattg tcgttagaac gcgtaatacg actcactata gggagag

47

<210> 8

<211> 47

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: XbaI cassette

<220>

<221> misc\_feature

<222> (47)

<223> complementary strand extends a single strand having

a sequence of 3'-gatc-5' at this position in the  
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<400> 8

gtacatattg tcgttagaac gcgtaatacg actcactata gggagat

47

<210> 9

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 9

cgtcttgcga ggattcagcg agctg

25

<210> 10

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 10

agctggattt cggccatgaa ttcta

25

<210> 11

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 11

gatctgttcg gtccgcaatc act

23

<210> 12

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 12

cactgggtgga gatgttcct cagat

25

<210> 13

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 13

catcttcgca accgcatcca tggcc

25

<210> 14

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 14

cgcgcagggt gcagcatgtt tggc

24

<210> 15

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 15

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25

<210> 16  
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 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 16  
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25

<210> 17  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 17  
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25

<210> 18  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for PCR

<400> 18  
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25

<210> 19  
 <211> 36  
 <212> DNA  
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: cassette  
 primer 1



36

$\langle 210 \rangle$	20
$\langle 211 \rangle$	35
$\langle 212 \rangle$	DNA
$\langle 213 \rangle$	Artificial Sequence

<220>  
<223> Description of Artificial Sequence: cassette  
primer 2

35

<210> 21  
 <211> 24  
 <212> DNA  
 <213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer for PCR

24

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10 Rev. 09 DEC 2002

## SEQUENCE LISTING

<110> IZUI, MASAKO  
SUGIMOTO, MASAKAZU  
KURAHASHI, OSAMU  
NAKAMATSU, TSUYOSHI

<120> DNA ENCODING SUCROSE PTS ENZYME II

<130> 217677US0PCT

<140> US 10/019,284

<141> 2002-01-02

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<151> 1999-07-02

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gaacccatgg tgttccttgt gggttgaggg aacgagtgcg ggtgagaagt ttttcaagtg	180
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Val Ser Trp Ile Leu Ala Thr Ile Glu Lys Phe Leu His Lys Arg Leu  
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Thr Gly Phe Leu Thr Phe Ile Ala Ile Gly Pro Ala Met Arg Trp Val  
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Lys Gln Gly Asp Glu Val Lys Ala Gly Glu Leu Leu Cys Glu Phe Asp  
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Ile Asp Ala Ile Lys Ala Ala Gly Tyr Glu Val Thr Thr Pro Ile Val  
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